A Morphological and Molecular Study of Bacterial Strains First Reported Locally in the City of Samarra and Isolated from Hydrocarbons Contaminated Soil

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Abstract

Background: Soil is a fundamental foundation for life on Earth; it is not merely a medium for plant growth and spread, but rather represents a complex biosystem with a diverse array of living organisms. Soil health and the absence of contamination particularly by hydrocarbons and their derivatives reflect the health of the entire ecosystem. Certain bacteria play a key biochemical role in the functioning of the ecosystem and in maintaining soil structure. Therefore, The aim of this study is to isolate and identify bacterial strains from hydrocarbon-contaminated soil in the city of Samarra at the morphological, microscopic, and molecular levels, and to identify any mutations in them, if present.

Methods: Soil samples were collected from two sites in the city of Samarra: the first site consisted of soil samples from power generators, and the second site consisted of soil samples from fuel filling stations during the period of November 2025, The bacterial culture media were prepared, diluted in a 10-fold series, and inoculated onto the media. The samples were then identified morphologically, microscopically, and using the D2MINI device, followed by molecular identification and sequencing of the 16S rRNA gene, and the results were analyzed.

Results: The results of bacterial identification from both soil types, following cultivation on Blood Agar and MacConkey Agar media, and based on colony morphology, cultural characteristics, microscopic examination, and identification using the D2 MINI Microbial ID/AST system, revealed the presence of *Bacillus licheniformis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* in both soil types.Molecular identification of these isolates was subsequently performed using the Polymerase Chain Reaction [PCR] technique targeting the 16S rRNA gene. Nucleotide sequencing results were compared with reference sequences available in the NCBI GenBank database. The findings demonstrated that the *Bacillus licheniformis* and *Bacillus subtilis* isolates investigated in this study represent novel strains recorded for the first time at the local level in Iraq due to the presence of nucleotide variations and mutations. The *Bacillus licheniformis* isolate exhibited six mutations with a sequence similarity of 99%, including five transversion mutations and one transition mutation. In contrast, the *Bacillus subtilis* isolate showed six mutations with a sequence similarity of 98%, comprising five transversion mutations and one insertion mutation. To document these novel strains, they were deposited in the NCBI GenBank database and assigned unique accession numbers for their nucleotide sequences as follows: accession number PZ694937 for *Bacillus licheniformis* strain Marwa1, and accession number PZ694938 for *Bacillus subtilis* strain Marwa2 and accession number PZ694939 for *Pseudomonas aeruginosa* strain Marwa 3.

Conclusion: Our study found that soil samples from power plants and fuel filling stations contaminated with hydrocarbons contained bacterial species that had acquired adaptive and acclimatory mutations to resist these pollutants, leading to the emergence of new strains for the first time locally in the city of Samarra. This was confirmed through DNA-based molecular diagnostic methods, which confirmed the presence of mutations in these strains, identified as *Bacillus licheniformis, Bacillus subtilis,* and *Pseudomonas aeruginosa*.

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Keywords: *Bacillus licheniformis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, Molecular diagnosis, Soil Contaminated, Hydrocarbons,

Introduction

Soil is a fundamental and vital foundation for life on Earth; it is one of the most important components of terrestrial ecosystems. It is not merely a medium for plant growth and spread, but rather a complex, thriving biosystem rich in biodiversity [1]. Soil health and the absence of contamination are a direct reflection of the health of the entire ecosystem. However, in the face of rapid urban, industrial, and agricultural expansion, soils worldwide are under enormous pressure, foremost among which is contamination by hydrocarbons [2]. In Iraq, soil contamination occurs due to the presence of hazardous chemicals or other changes in the natural soil environment. This problem is primarily attributed to industrial emissions, the excessive use of agricultural inputs including insecticides, herbicides, and fertilizers as well as the leakage of pollutants from waste sites and the runoff of contaminated water. The most common pollutants include heavy metals and petroleum hydrocarbons [3,4].

Hydrocarbon derivatives produced primarily by the oil and gas industry, such as oils, fuels, greases, and refining byproducts are hazardous organic pollutants that contaminate soil and are widely distributed [5]. The presence of petroleum hydrocarbons in soil has a significant and direct impact on soil biological properties, as these compounds cause severe damage to the soil microbial community. Through various mechanisms, they damage the outer cell membrane of microorganisms, It leads to changes in the fluidity of the cell membrane and alterations in its functions, thereby inhibiting many of the vital metabolic activities and functions of microorganisms. [6]. Field studies have shown that contamination with crude oil and its derivatives leads to a significant and noticeable decline in soil biodiversity and microbial diversity. This has been confirmed by research indicating that soil contaminated with gasoline and diesel exhibited a significant reduction in the diversity of bacterial microbial communities, demonstrating the harmful effects of hydrocarbons and their derivatives on soil microflora [7].

Certain bacteria play a key biochemical role in ecosystem functioning through the transformation of organic matter, decomposition, nutrient cycling, and the maintenance of soil structure, as well as the vast majority of biochemical processes in both aquatic and terrestrial habitats [8]. Numerous microbial organisms capable of degrading petroleum and its derivatives have been isolated and tested, including the genera *Achromobacter, Acinetobacter, Alcaligenes, Pseudomonas, Arthrobacter, Bacillus,Corynebacterium,Brevibacterium, Flavobacterium, Micrococcus, Nocardia, and Streptomyces and Rhodococcus* [9]. The bacteria *Bacillus* sp., *Pseudomonas aeruginosa,* and *Rhodococcus erythropolis* were isolated from diesel-contaminated soil and achieved an 85% efficiency in degrading diesel fuel within seven weeks [10].

The study include identified isolates of species morphologically and confirmed their identification genetically after being isolated and purified.

Material and Methods

Study Location The study was conducted in the laboratories of the College of Education/Department of Life Sciences at the University of Samarra from November

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1, 2025, to June 10, 2026. Two hydrocarbon-contaminated sites were chosen. The first site was was the area where the generators were located, and the second was the fuel filling stations. in the city of Samarra.

Sample Collection

The soil samples under study were collected from two locations using a Soil Auger to a depth of 15 cm after removing vegetation and stones, if present in the city of Samarra. The first location involved collecting soil samples from areas near power generators, and the second location from which the soil samples under study. Remove plants and stones from the soil, then till the soil to a depth of 15 cm.. The soil samples were placed in sterile, airtight paper bags, labeled with the sample name, collection site, collection date, and quantity, and sent to laboratories specializing in soil analysis, including the laboratories of the General Company for the Manufacture of Medicines and Medical Supplies in Samarra and the Sciences Laboratories at the College of Education, University of Samarra.

Preparing the Culture Media

Culture media were prepared according to the manufacturer's instructions (HiMedia, India), which included solid media such as MacConkey agar and blood agar; the pH was adjusted to 7. The media were sterilized using an autoclave, and 12 isolates were cultured on these media. They were identified morphologically and microscopically, as well as through biochemical testing using the D2MINI device, which includes 26 biochemical tests.**Identification of Bacterial Isolates**

After observing the growth of bacterial colonies on the culture media, these isolated colonies were purified by initial inoculation onto pre-prepared culture media (blood agar and MacConkey agar) media. The purified bacteria were then Morphological Characterized and identified through and microscopically examination identified after being stained separately using the Gram stain. identified after being stained separately using the Gram stain. Following the initial identification, the bacterial isolates were identified at the species level using the D2 MINI Microbial ID/AST system, which includes several biochemical tests, such as the oxidase test, the catalase test, the indole test, the citrate test, the methyl red test, the hemolysis test, and the triglyceride test tailored to each bacterium and performed according to the manufacturer's instructions [12,13,14].

Molecular Diagnosis of Bacterial Isolates

A molecular study was conducted to identify three different bacterial isolates from bacteria isolated from hydrocarbon-contaminated soil. using the Presto™ Mini gDNA Bacteria Kit from Geneiad (Taiwan).and the concentration and purity of the DNA were determined using a Nanodrop instrument.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was performed using specific primers to amplify the 16S rRNA gene (Table 1).

Table 1: 16S rRNA gene primers

Primer	Sequence (5'-3'direction)	GC٪	Reference
16s rRNA	**F-5`- GTGCCAGCAGCCGCTAA-3` R-3`-AGAACCCGGGAACGTATTCAC-5`**	**64.71 52.38**	**Miller *et al.*,(2013)**

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The reaction was carried out in a total volume of 25µl according to the steps outlined in Table 2.

Table 2: PCR Cycle Protocol

Program steps	Temperature ^{0}C	Time	No.of cycle
Initial denaturing	**95**	**3 min**	**1**
Denaturing **Annealing** **Extension**	**95** **50** **72**	**30 min** **30 sec** **1 min**	**30**
Final extension	**72**	**5 min**	**1**

Agarose Gel Electrophoresis

Electrophoresis was performed using 2% agarose gel to verify the PCR products, following the method described by [16] The nucleotide sequences of the 16S - rRNA gene, which had been amplified in bacterial isolates from hydrocarbon-contaminated soil, were analyzed by sending them to Macrogen Corporation for processing using a genetic analyzer. The results were then analyzed and compared with the NCBI gene databaseTheThe Based on the identification of more than one nucleotide mutation in each bacterial species, these isolates were considered new strains recorded for the first time and were documented in a Gene Bank.

Phylogenetic tree analyses

The phylogenetic tree for each bacterial species was analyzed in relation to the species listed globally in NCBI, using the BLAST tool and selecting the neighbor joining icon.

Results and Discussion

The results in Table 1 show the bacterial isolates collected from hydrocarbon-contaminated soil in the city of Samarra, including two sites: the first site being soil from power generators and the second site being soil from fuel filling stations, These were identified macroscopically and microscopically, as well as through biochemical tests using the D2 MINI Microbial ID/AST device. The study identified the following bacterial species: *Bacillus licheniformis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* in both types of soil studied.

Table 1: Bacterial Isolates from Generator Soil and Fuel Filling Station Soil

Bacterial Isolation	Gram stain	Cell Shape
Bacillus licheniformis	Gram-positive	Spore-forming bacillus
Bacillus subtilis	Gram-positive	Spore-forming bacillus
Pseudomonas aeruginosa	Gram-negative	Rod-shaped

Isolates of *Bacillus* bacteria (B.*licheniformis and B. subtilis*) Based on the morphological and microscopic characteristics observed in culture media, these isolates grew on blood agar and

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formed colonies that were white with a grayish tinge, with a rough surface and irregular edges; they also tested positive for Gram stain and appeared rod-shaped under the microscope. (Figures 1 and 2). These morphological characteristics are consistent with studies that examined the morphological and microscopic characteristics of this genus, including those by [17] and [18].

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Figure 1: Morphological and Microscopic characters of *B. licheniformis* on the Blood agar plate

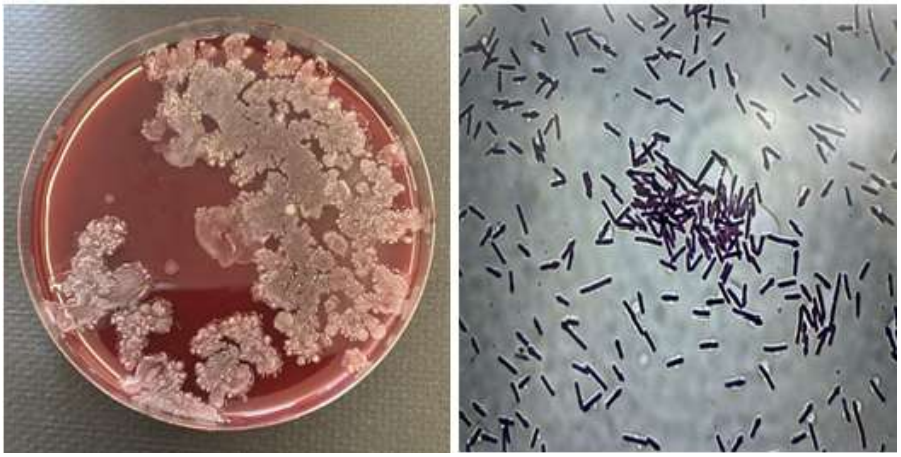


Figure 2: Morphological and Microscopic characters of *B. subtilis* on the Blood agar plate

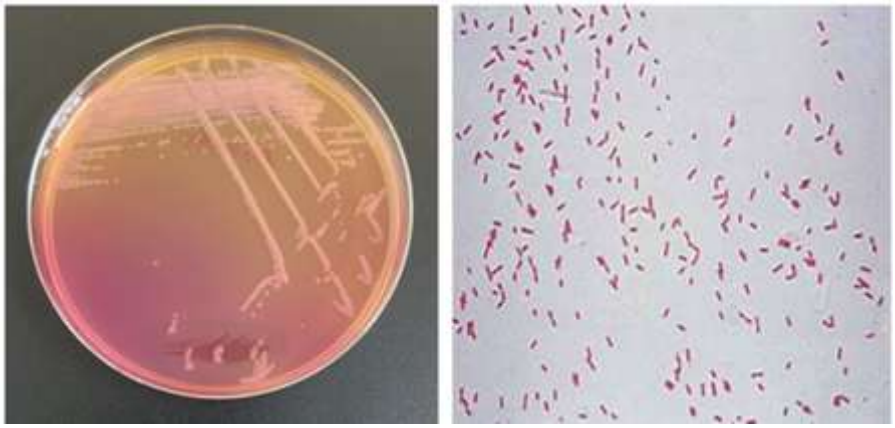


Figure 3: Morphological and Microscopic characters of *P. aeruginosa* on MacConkey agar plate

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The results of this study revealed the isolation and identification of the bacterial species *Bacillus licheniformis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* at both soil sites, the first of which included power generators and the second fuel filling stations. These results are attributable to the fact that bacterial species belonging to the genera *Bacillus* and *Pseudomonas* are globally known for their remarkable ability to tolerate, resist, metabolize, and biodegrade hydrocarbons in contaminated environments. Consequently, the isolation of these genera from the two sampling sites is most likely due to the common hydrocarbon contamination shared by these two soil types. This is consistent with a local study in Iraq that found these bacterial species could be isolated from the soil of fuel filling stations and electrical generators [21], The widespread prevalence of bacterial species belonging to the genera *Pseudomonas* and *Bacillus* may be attributed primarily to their possession of A molecular study of the results of the The current results of a molecular study revealed successful DNA extraction of purified isolates (Table 2) show the results of measuring the purity and The DNA concentration of the bacterial isolates enzymes specialized in hydrocarbon oxidation, particularly dioxygenases and oxygenases, These species can also utilize petroleum derivatives as a source of energy and carbon, in addition to their tolerance for harsh environmental conditions and their resistance to pollutants, including heavy metals, which gives them a competitive advantage in contaminated soils [21,22]. They also exhibit high tolerance to harsh environmental conditions and various pollutants, including heavy metals, providing them with a competitive advantage in hydrocarbon-contaminated soils [22]. Furthermore, the occurrence of *B. licheniformis* and B. subtilis in both sampling sites in this study in this study may be explained by the fact that the genus Bacillus is widely recognized as one of the most abundant and well-adapted bacterial genera inhabiting petroleum-contaminated soils worldwide. This observation is supported by the study of [23]

under study was measured using a Nanodrop instrument; the purity ranged from 1.80 to 1.91, while the concentration of the samples ranged from 72.3 to 121.9 nanograms/mL.

Tabel 2: DNA Concentration and Purity of Bacterial Isolates

Purity [260/280]	**DNA Concentration (ng/ml)**	**NO.**
1.87	139	A1(*B. licheniformis)*
1.91	121.9	A2(***B. subtilis***)
1.80	72.3	B (*P. aeruginosa*)

The purity and concentration of DNA are very important in genetic studies, since the purity and concentration of DNA affect other molecular processes; the higher the purity and the more appropriate the concentration, the better and more accurate the results of subsequent steps will be. Studies have shown that the presence of impurities in DNA hinders subsequent molecular steps, particularly the impurity-sensitive PCR reaction, as well as the DNA sequencing step [24,25]. DNA purity is of great importance, as it is an essential requirement for avoiding chemical inhibition of the polymerase chain reaction [26].

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Electrophoresis of the 16S rRNA gene amplification products revealed bands at 800 bp for all bacterial isolates. revealed. This gene was amplified via polymerase chain reaction (PCR) using specific primers, Bands appeared for all the appearance of these bands at this molecular size indicate that the primers specific to the target gene were bound and that amplification occurred, as shown in Figure 4.

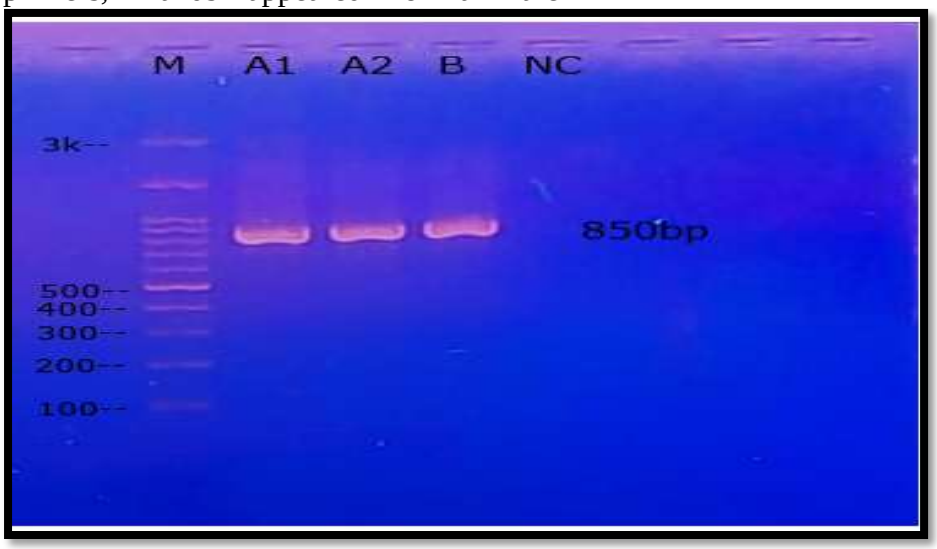


Figure 4: Electrophoresis of the 16S rRNA gene after PCR amplification on a 1.5% agarose gel at 70 volts for 1.5 hours for bacterial isolates.

The results of the 16S rRNA gene amplification and PCR analysis were consistent with [27], which demonstrated that the 16S rRNA gene is highly sensitive and serves as an important diagnostic tool for distinguishing between bacterial strains. These results were also consistent with [28], who were able to identify bacterial isolates at the species and strain levels from soil samples contaminated with hydrocarbons using the 16S -rRNA gene, demonstrating that this gene is important for identifying bacterial species because it is highly sensitive, conserved within the bacterial genome, and crucial for distinguishing between genera at the species and strain levels. The results of our study also concurred with those of [30], who were able to identify numerous bacterial strains and species from soils contaminated with diesel and hydrocarbons—including the genera Pseudomonas and Bacillus—using the 16S rRNA gene. The reason for using this gene in molecular identification is that study [31] demonstrated that the 16S rRNA gene remains, to a large extent, a decisive and primary marker for identifying microbial organisms, It is also one of the most extensively studied sequences ever, and this gene serves as a variable marker capable of distinguishing organisms down to the strain level; it acts as a living fossil carrying information about the origins of cellular life.

After comparison with the sequences in NCBI GenBank, the results showed that the sequences belonged to the *Bacillus licheniformis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa.*

These results were consistent with phenotypic and microscopic identification, as well as with identification using the D2 MINI Microbial ID/AST system. Furthermore, molecular diagnosis through

The results presented in Table (3) show the alignment of the sequences of the isolates under study with sequences in NCBI; the highest match was 100% with

Pseudomonas aeruginosa registered in Iraq under ID: MZ950650.1, and the lowest match rate was 98% with *Bacillus subtilis* registered in China under ID: FJ392725.1. The species *Bacillus licheniformis* showed a 99% match with sequences registered in Turkey under ID: D: PX498602.1, with 6 mutations.

Table 3: Results of a comparison of bacterial isolates sequenced from soil samples taken from power generators and fueling stations with the most closely related sequences in NCBI.

Identities	Strain	Country	Sequence ID with compare	Substitution mutation	Type of substitution	No
99 %	***Bacillus licheniformis***	**Turkey**	**ID:PX498602.1**	**C/G**	**Transvertion**	**1**
				C/A	**Transvertion**	
				G/T	**Transvertion**	
				T/G	**Transvertion**	
				C/A	**Transvertion**	
				T/C	**Transition**	
98%	***Bacillus subtilis***	**China**	**ID:FJ392725.1**	**Insertion A**	**Insertion**	**2**
				G/T	**Transvertion**	
				C/A	**Transvertion**	
				A/C	**Transvertion**	
				C/G	**Transvertion**	
				C/G	**Transvertion**	
100%	***Pseudomonas aeruginosa***	**Iraq**	**ID: MZ950650.1**	**-**	**-**	**3**

Figure 5 shows the sequence alignment for *Bacillus licheniformis*, with a 99% match and six mutations; Figure 6 shows the sequence alignment for *Bacillus subtilis*, with a 98% match and six mutations; and Figure 7 shows the sequence alignment for *Pseudomonas aeruginosa*, with a 100% match.

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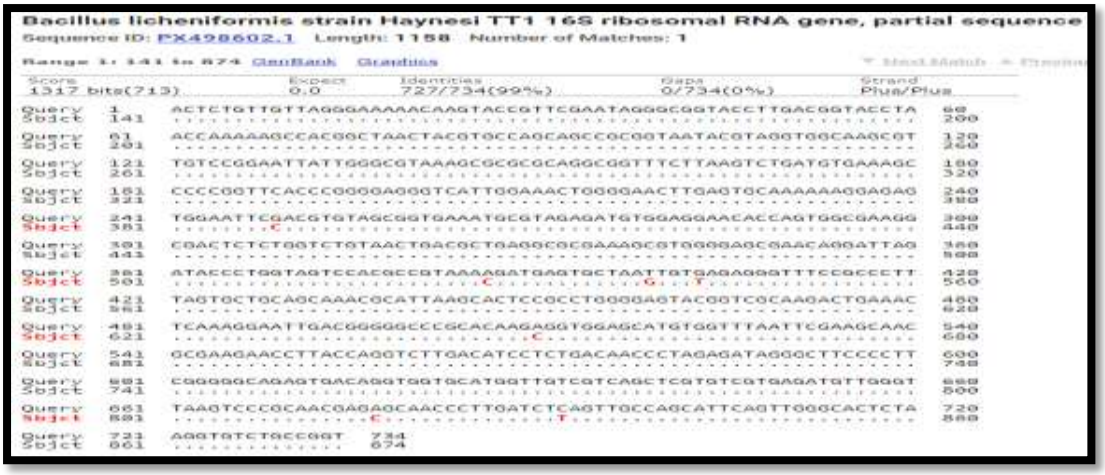


Figure 5: Alignment of the *Bacillus licheniformis* sequence in BLAST.

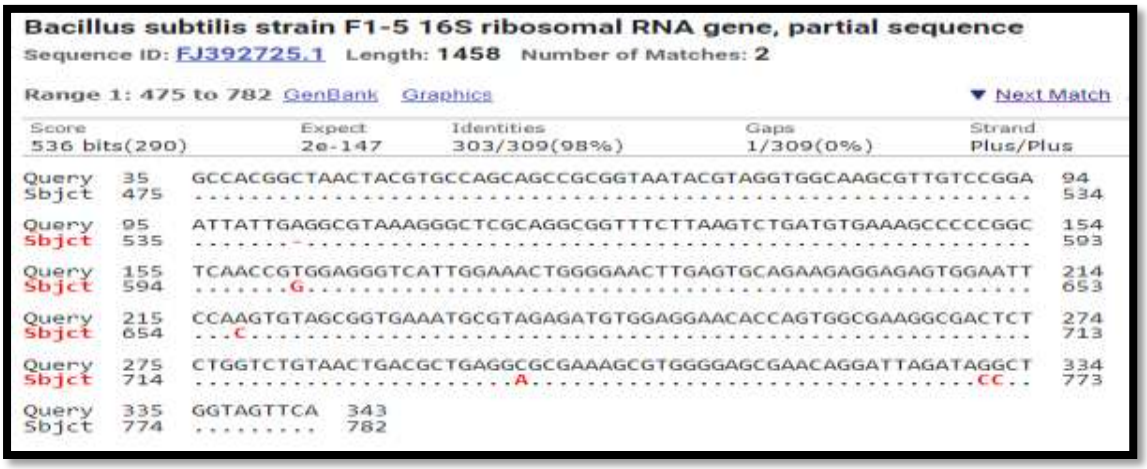


Figure 6: Alignment of the *Bacillus subtilis* sequence in BLAST

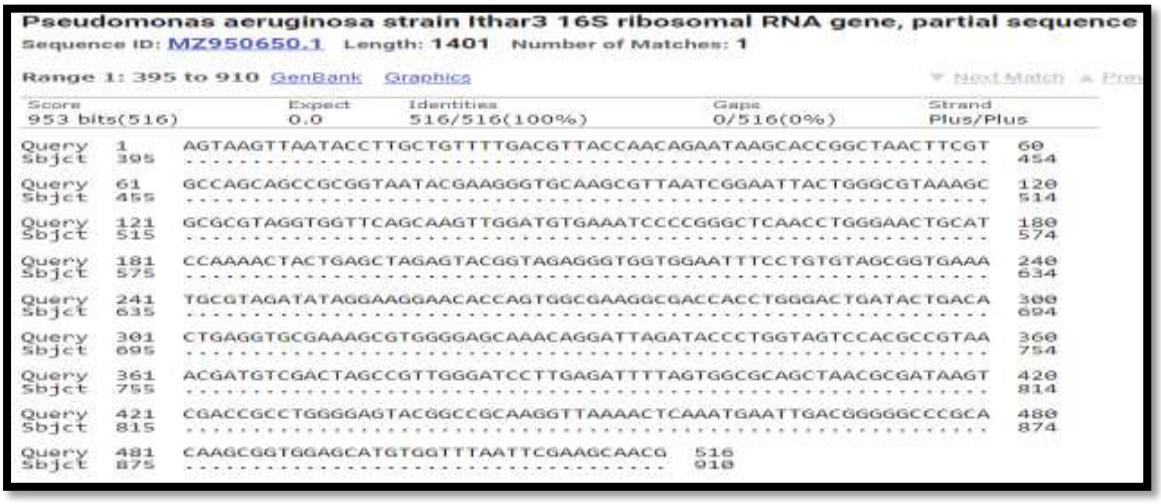


Figure 7: Alignment of the *Pseudomonas aeruginosa* sequence in BLAST

As for the registration of new strains of the species Bacillus licheniformis and Bacillus subtilis in the NCBI GenBank database, and given the presence of mutations and genetic variations in them, this may be due to the continuous, long-term exposure of these bacteria to environmental stresses in soil

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contaminated with hydrocarbons and their derivatives at power plants and fuel filling stations; These soils contain a complex mixture of aromatic and aliphatic hydrocarbons, in addition to toxic compounds and heavy metals, all of which can act as selective pressures on these bacterial species, leading to the accumulation of genetic variations over time, The cause of these mutations may be an adaptive mechanism that allows these bacterial species to resist and utilize hydrocarbons and their derivatives as a source of energy and carbon, granting them a selective advantage and competitive edge compared to other bacterial species in uncontaminated soils[31], The cause of these mutations may also be attributed to horizontal gene transfer, which is considered the most prominent feature in bacterial evolution; horizontal gene transfer and positive selection may lead to the emergence of specific, detectable markers in genomes. [32] Studies have also confirmed that genetic mutations, which provide the raw material for evolutionary adaptation, are largely a random force; however, mutations can also exhibit strong biases, as certain types of mutations and specific genomic loci mutate at a higher rate than others. It has become increasingly clear that mutation bias can play a role in determining adaptive outcomes in bacteria [33]. A recent study has shown that environmental conditions and stress on bacterial species cause oxidative stress, which in turn disrupts the genetic material and genome of these bacteria, resulting in bacterial genome rearrangement and horizontal gene transfer caused by oxidative damage to cellular components. Thus, it can be said that the rapid colonization of new nutrient substrates and ecological niches by microorganisms occurs due to accelerated genetic changes caused by oxidative stress, mediated by several stress-regulatory genes (SOS, RpoS, and RpoE) and under selective pressure, which may result in genetic changes and new mutations [34]

The phylogenetic trees of bacterial were analyzed using the Mega software, based on the nucleotide sequences of the bacterial species recorded in the NCBI GenBank. This analysis was conducted to determine the similarities and differences among the species; the phylogenetic tree illustrates a phylogenetic relationship among the species, as shown in Figures 8, 9, and 10.

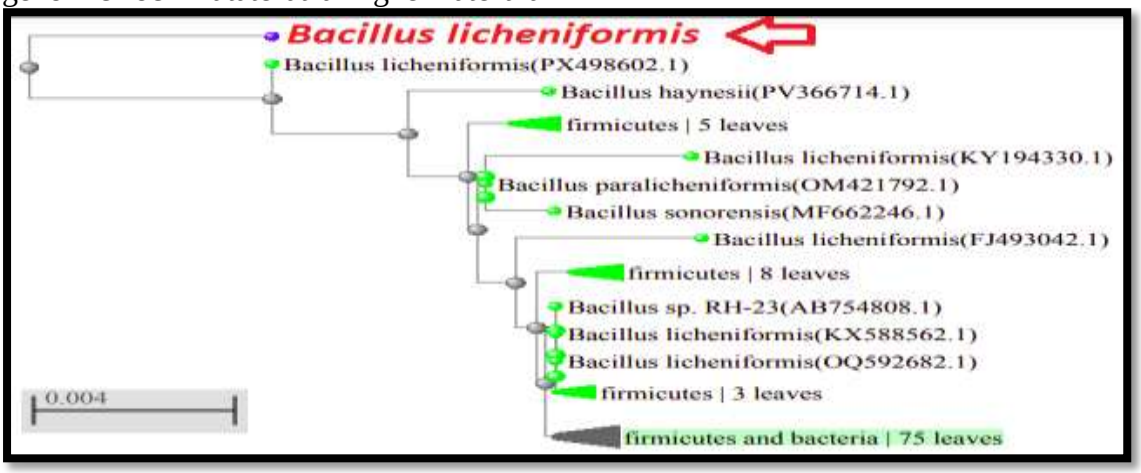


Figure 8: Phylogenetic tree of the *Bacillus licheniformis* using the Mega software.

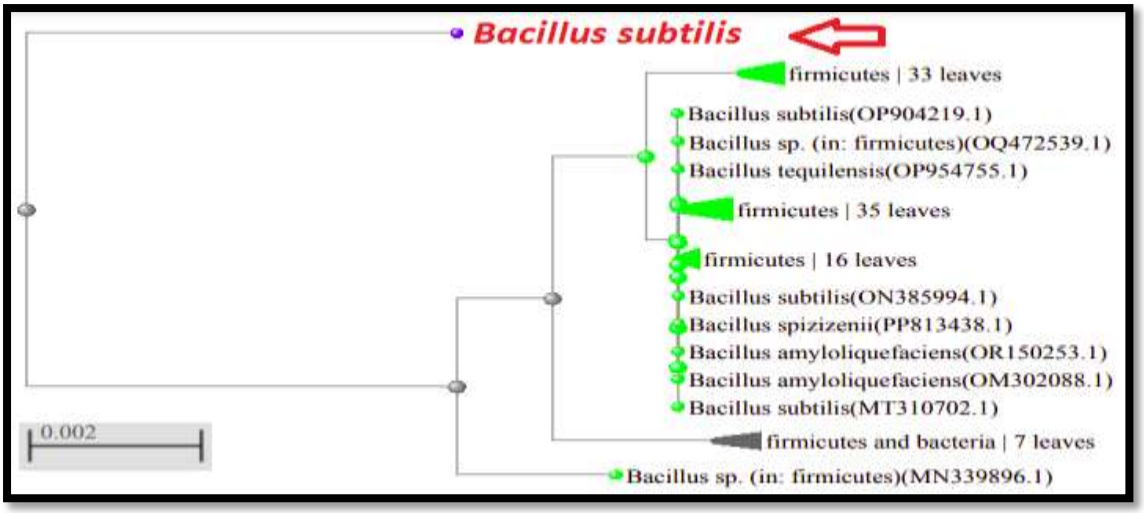


Figure 8: Phylogenetic tree of the *Bacillus subtilis* using the Mega software.

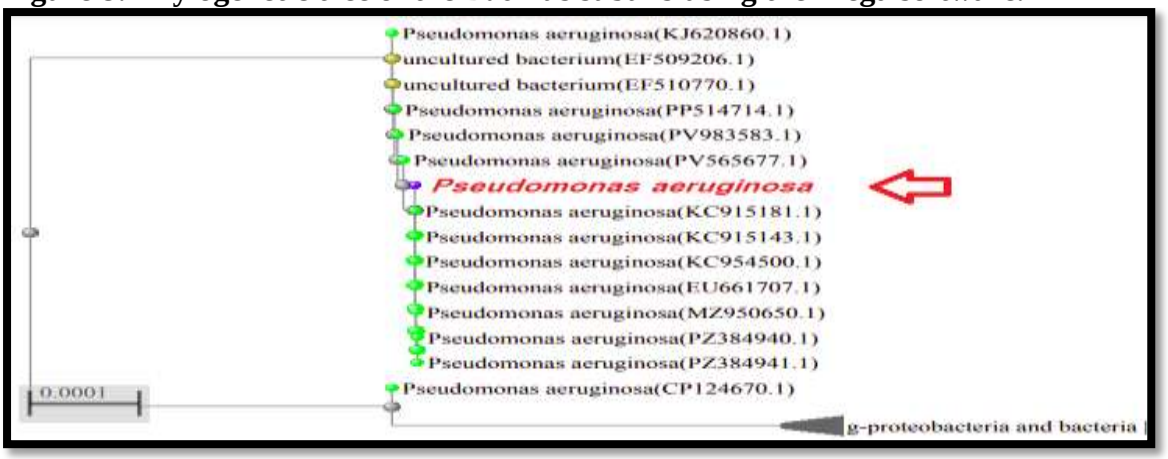


Figure 8: Phylogenetic tree of the *and P. aeruginosa* using the Mega software

Studying the phylogenetic tree is important for understanding the genetic and evolutionary relationships among species; it is also essential for understanding and studying the evolution of mechanisms associated with virulence factors and for examining ecological life histories. Genetic and phylogenetic analyses can be conducted using several tools based on the alignment of nucleotide sequences between species. For example, 16S rRNA gene sequences have been used to understand the phylogenetic relationships among bacterial species isolated from houseflies, enabling the determination of the species' evolutionary history and diversity characteristics, the prediction of gene novelty, and the understanding of the closeness or distance between pathogenic microbial strains, among other purposes [22,35]

After completing the tabulation and analysis of the nitrogen-fixing gene sequence results for all bacterial isolates under study, and in order to document the strains reported for the first time at the local level, these results were documented by depositing all sequences in the NCBI GenBank, where each strain was assigned a unique accession number, as shown in Table

Table 7: GenBank Accession Numbers for the Bacterial Strains Under Study

Accession Number	Strain
ID:PZ694937	*Bacillus licheniformis* strain Marwa1
ID:PZ694938	*Bacillus subtilis* strain Marwa 2
ID:PZ694939	*Pseudomonas aeruginosa* strain Marwa 3

Conclusion

Our study found that soil samples from power plants and fuel filling stations contaminated with hydrocarbons contained bacterial species that had acquired adaptive and acclimatory mutations to resist these pollutants, leading to the emergence of new strains for the first time locally in the city of Samarra. This was confirmed through DNA-based molecular diagnostic methods, which confirmed the presence of mutations in these strains, identified as *Bacillus licheniformis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.

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